Title

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Journal

Journal of veterinary internal medicine, 32(4)

ISSN

0891-6640

Authors

Smith, Fauna Leah
Watson, Johanna L
Spier, Sharon J
et al.

Publication Date

2018-07-01

DOI

10.1111/jvim.15145

Peer reviewed

Received: 16 October 2017 | Revised: 20 February 2018 | Accepted: 12 April 2018

DOI: 10.1111/jvim.15145

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Frequency of shedding of respiratory pathogens in horses recently imported to the United States

Fauna Leah Smith[1] | Johanna L. Watson[2] | Sharon J. Spier[2] | Isabelle Kilcoyne[3] | Samantha Mapes[2] | Claudia Sonder[4] | Nicola Pusterla[2]

[1]Willian R Pritchard Veterinary Medical Teaching Hospital, University of California Davis, Davis, California

[2]Department of Medicine and Epidemiology, University of California Davis, Davis, California

[3]Department of Surgical and Radiological Sciences, University of California Davis, Davis, California

[4]Center for Equine Heath, School of Veterinary Medicine, University of California, Davis, Davis, California

Correspondence
Fauna Leah Smith, Graduate Group in Integrative Pathobiology, Center For Comparative Medicine, University of California, Davis, CA 95616.
Email: flsmith@ucdavis.edu

Funding information
University of California; Davis Center for Equine Health; State of California Satellite Wagering Fund; Private Donors

Background: Imported horses that have undergone recent long distance transport might represent a serious risk for spreading infectious respiratory pathogens into populations of horses.

Objective: To investigate the frequency of shedding of respiratory pathogens in recently imported horses.

Animals: All imported horses with signed owner consent (n = 167) entering a USDA quarantine for contagious equine metritis from October 2014 to June 2016 were enrolled in the study.

Methods: Prospective observational study. Enrolled horses had a physical examination performed and nasal secretions collected at the time of entry and subsequently if any horse developed signs of respiratory disease during quarantine. Samples were assayed for equine influenza virus (EIV), equine herpesvirus type-1, −2, −4, and −5 (EHV-1, −2, −4, −5), equine rhinitis virus A (ERAV), and B (ERBV) and *Streptococcus equi* subspecies *equi* (*S. equi*) using quantitative PCR (qPCR).

Results: Equine herpesviruses were detected by qPCR in 52% of the study horses including EHV-2 (28.7%), EHV-5 (40.7%), EHV-1 (1.2%), and EHV-4 (3.0%). Clinical signs were not correlated with being qPCR-positive for EHV-4, EHV-2, or EHV-5. None of the samples were qPCR-positive for EIV, ERAV, ERBV, and *S. equi*. The qPCR assay failed quality control for RNA viruses in 25% (46/167) of samples.

Conclusions and Clinical Importance: Clinical signs of respiratory disease were poorly correlated with qPCR positive status for EHV-2, −4, and −5. The importance of γ-herpesviruses (EHV-2 and 5) in respiratory disease is poorly understood. Equine herpesvirus type-1 or 4 (EHV-1 or EHV-4) were detected in 4.2% of horses, which could have serious consequences if shedding animals entered a population of susceptible horses. Biosecurity measures are important when introducing recently imported horses into resident US populations of horses.

KEYWORDS
biosecurity, equine herpesvirus, transport

1 | INTRODUCTION

Recently imported horses are likely to shed infectious respiratory pathogens, presenting a risk to resident horses in the United States. Surveillance studies have shown that equine influenza virus (EIV) and

Abbreviations: CEM, contagious equine metritis; DNA, deoxyribonucleic acid; EIV, equine influenza virus; EHV-1, equine herpesvirus type-1; EHV-2, equine herpesvirus type-2; EHV-4, equine herpesvirus type-4; EHV-5, equine herpesvirus type-5; ERAV, equine rhinitis A virus; ERBV, equine rhinitis B virus; qPCR, real time qualitative polymerase chain reaction; RNA, ribonucleic acid; *S. equi*, *Streptococcus equi* subsp. *equi*; WBC, white blood cell.

equine herpesvirus type-4 (EHV-4) continue to be prevalent viral respiratory pathogens throughout the United States and Europe.[1,2] These highly contagious and virulent respiratory viruses have major financial implications for the equine industry.[3,4]

Long distance horse transportation can result in substantial stress and alteration in immune function, including an acute phase response that results in impaired cell-mediated immunity.[5,6] Imported horses also have increased exposure risk to infectious respiratory pathogens, because of comingling at sales/shipping barns, quarantine facilities, and during transports.

The goal of our study was to determine the detection frequency of selected infectious respiratory pathogens from horses recently imported to the United States. Specifically, we analyzed nasal swabs for shedding of EIV, equine herpesvirus type-1 (EHV-1), equine herpesvirus type-2 (EHV-2), equine herpesvirus type-4 (EHV-4), equine herpesvirus type-5 (EHV-5), *Streptococcus equi* subsp. *equi (S. equi)*, equine rhinitis A virus (ERAV), and equine rhinitis B virus (ERBV).

2 | MATERIALS AND METHODS

2.1 | Animals

All horses with owner consent (n = 167) admitted to the contagious equine metritis (CEM) quarantine facility at the Center for Equine Health, University of California at Davis for CEM quarantine purposes were enrolled in our study from October 10, 2014 to June 30, 2016. The USDA approved facility quarantines ~200 recently imported horses on an annual basis. Examination and sample collection was performed ~72 hours after having arrived in the United States. Horses generally spent 48 hours in USDA import quarantine upon arrival at Los Angeles International Airport, 12 hours in transit to the CEM quarantine facility, and 12–18 hours in CEM quarantine before sampling.

2.2 | Sample collection

As part of the routine quarantine protocol, horses entering the quarantine facility had a physical examination performed and blood collected for a complete blood cell count. The physical examination and blood draw was performed by a veterinarian from the Equine Field Service of the UC Davis VMTH. Horses were defined as having clinical signs associated with respiratory disease if they had any one: nasal discharge, tachypnea (respiratory rate >24 breaths per minute), fever (>101.5°F), cough, submandibular or retropharyngeal lymph node enlargement, or abnormal lung sounds. Data for hematocrit, white blood cell counts, and fibrinogen were collected from the complete blood counts for each horse. Additionally, 6″ rayon-tipped nasal swabs were collected at the time of entrance from each horse with informed owner consent. Horses that developed lethargy, anorexia, fever, excessive nasal discharge, lymphadenopathy, or cough during the quarantine period (14 days for mares, 31 days for stallions) had additional nasal swabs collected at the time of the onset of clinical signs and the pertinent physical exam changes recorded. The swabs were transferred to tubes containing viral transport medium (Viral transport media—provided by laboratory bovine calf serum [62.5 mL], minimum essential media [500 mL], Penicillin/Streptomycin [5 mL], and Gentamicin [500 μL]) and transported to the laboratory (Real-time PCR Research and Diagnostics Core Facility, School of Veterinary Medicine, Department of Medicine and Epidemiology, 3110 Tupper Hall, University of California, Davis, CA 95616) for sample processing and analysis. The nasal secretion samples were stored at 4°C until being processed and analyzed within 96 hours of collection.

2.3 | Sample processing and PCR analysis

Nucleic acid extraction from nasal secretions was performed using an automated nucleic acid extraction system (CAS-1820 X-tractor Gene, Corbett Life Science) according to the manufacturer's recommendations. Total RNA was purified from nasal secretions as follows: 20 μL of each freshly extracted nucleic acid sample from nasal secretions (containing genomic DNA [gDNA] and total RNA) was digested with DNAse for 60 minutes at 37°C to remove gDNA. DNase was inactivated at 95°C for 5 minutes. Complementary DNA from each sample was synthesized using 50 U SuperScript III (Invitrogen) in a 40 μL final volume containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 0.5 mM dNTPs, 40 U RNAsin, 0.5 mM dithiothreitol, and 600 ng random hexadeoxyribonucleotide (pd [N] 6) primers (random hexamers, Invitrogen). The reaction was performed at 50°C for 60 minutes. After inactivation at 95°C for 5 minutes, the reaction volume was adjusted to 100 μL with nuclease-free water.

Nasal secretions were assayed for the presence of the EIV, EHV-1, EHV-2, EHV-4 EHV-5, *S. equi*, ERAV, and ERBV using previously reported quantitative TaqMan PCR assays.[1,7] To determine the sample quality and efficiency of nucleic acid extraction all samples were analyzed for the presence of the housekeeping gene *equine glyceraldehyde-3-phosphate dehydrogenase* as previously described.[8] The laboratory defines failure of quality control as inadequate genomic material or the presence of inhibitors of the qPCR reaction in samples.

2.4 | Data analysis

Most of the data is presented as descriptive results with population proportions, percentages, or both being reported. Odds ratios were calculated for differences between breed groups. Numerical data was analyzed for normality using the Shapiro-Wilk test. Hematologic variables were normally distributed and were compared using a one-way ANOVA (VassarStat, Online version, Vassar College, Poughkeepsie, New York). Horses were split into four groups for comparison of hematologic variables: PCR-positive nasal swab/respiratory disease clinical signs positive, PCR-negative nasal swab/respiratory disease clinical signs negative, PCR-positive nasal swab/respiratory disease clinical signs negative, and PCR-negative nasal swab/respiratory disease clinical signs positive. A *P*-value of significance was set at <.05.

3 | RESULTS

One hundred sixty-seven individual horses were enrolled in the study. The median age was 7 years with a range of 3 weeks-21 years. There were no yearlings; however, there were 3 foals under the age of 1 year (range 1–6 months) that were accompanying their dams. The population included 161 mares, 6 stallions and no geldings. Breed groups represented were European warmbloods (79.0%; 132/167), Friesians (8.4%; 14/167), baroque breeds (7.8%; 13/167), ponies (1.8%; 3/167), and others (3.0%; 5/167). All of the horses in our study entered from Europe with the majority coming from Germany (34.7%; 58/167), The Netherlands (29.3%; 49/167), and Belgium (8.4%; 14/167) with smaller percentages from Spain (6.0%; 10/167), The United Kingdom (4.8%; 8/167), France (4.8%; 8/167), Ireland (3.6%; 6/167), Denmark (2.4%; 4/167), Portugal (1.8%; 3/167), Italy (1.8%; 3/167), Sweden (1.2%; 2/167), Switzerland (0.6%; 1/167), and Austria (0.6%; 1/167).

3.1 | Herpesviruses

γ-Herpesviruses were more commonly recovered than α-herpesviruses. Equine herpesvirus type-2 was found in 27.7% (48/167) and EHV-5 was recovered from 40.7% (68/167) of horses. Of horses shedding EHV-2 and EHV-5, 58.3% (28/48) and 64.7% (44/68) were asymptomatic, respectively. Of horses shedding EHV-5, 38.2% (26/68) were shedding viral loads greater than 650 000 gB genes/million cells and of those horses 65.4% (17/26) were asymptomatic, similar to the percentage in the entire EHV-5 PCR-positive population. The clinical signs in horses, qPCR positive for γ-herpesvirses are summarized in Table 1.

The gB gene for EHV-1 non-neuropathogenic (A2254 genotype) was detected in nasal secretions from 1/167 horses on arrival (a 3-week old filly) and in one additional animal (her dam) that developed clinical signs during the quarantine period. Both the foal and her mare had consistent clinical signs of tachypnea (respiratory rate 90–100 breaths per minute), mucoid nasal discharge, and fever (102.0–102.5°F; Table 1). The viral load was high in the filly with 1.4×10^7 EHV-1 gB genes/million nasal cells. That filly was also positive in whole blood for the EHV-1 gB genes (9.7×10^2 gB genes/million WBCs). The filly's dam was positive in low numbers in nasal swab associated with clinical signs of respiratory disease (5.1×10^3 gB genes/million nasal cells) but qPCR-negative in blood. These horses were Andalusians from Spain. Both horses were still positive in nasal secretions at the time of discharge from quarantine, approximately 14 days (filly) and 11 days (dam) after onset of clinical signs, with recommendations to strictly quarantine at their destination. Ultimately, 1.2% of the population was shedding EHV-1 (A2254 genotype). Equine herpesvirus type-1 neuropathogenic (G2254 genotype) was not detected from any of the horses in our study.

TABLE 1 Clinical signs: Summary of clinical signs seen in horses qPCR positive for herpesviruses

Clinical signs	EHV-1 (n = 2)	EHV-4 (n = 5)	EHV-2 (n = 48)	EHV-5 (n = 68)
Fever (n = 11)	2	2	2	5
Tachypnea (n = 9)	2	1	1	5
Cough (n = 5)	0	0	2	3
Enlarged lymph nodes (n = 11)	0	0	6	5
Abnormal lung sounds (n = 0)	0	0	0	0
Nasal discharge (n = 42)	2	1	16	23
No clinical signs (n = 74)	0	2	28	44

Equine herpesvirus type-4 was detected in 4/167 horses on arrival at the quarantine facility and in one additional animal that developed a fever during the quarantine period. In our study, 3.0% of horses in our study were shedding EHV-4. Of the horses shedding EHV-4, 60.0% (3/5) had clinical signs associated with respiratory disease and 40.0% (2/5) were asymptomatic (Table 1). Friesians were more likely to shed EHV-4 than European warmbloods (OR 7.17 [1.09–47.18] $P = .041$). The EHV-4 positive horses came from The Netherlands (2), Germany (1), the United Kingdom (1), and Ireland (1). There was no significant effect of country of origin on EHV-4 shedding.

All three foals enrolled in the study were qPCR-positive for herpesviruses on nasal swabs: EHV-1 (1), EHV-2 (2), and EHV-5 (2). All of the foals were shedding viral loads greater than 1.8×10^6 gB genes/million cells regardless of the type of herpesvirus. As a comparison, the median viral load for horses ≥2 years was 1.8×10^4 gB genes/million cells. Two foals had coinfections (EHV-1/EHV-5 and EHV-2/EHV-5) and one foal was only positive for EHV-2. The two foals with coinfections both had clinical signs associated with infectious respiratory disease, whereas the foal shedding only EHV-2 was asymptomatic.

Twenty-eight adult horses were qPCR positive for multiple herpesviruses: EHV-2 and EHV-5 (25/28), EHV-1 and EHV-5 (1/28), and EHV-2, EHV-4, and EHV-5 (2/28). In horses with co-infections, 42.9% (12/28) had clinical signs associated with respiratory disease and 57.1% (16/28) were asymptomatic.

Of the nasal swab samples submitted for herpesvirus qPCR, 6.0% (10/167) for α-herpesviruses and 6.6% (11/167) for γ-herpesviruses, failed quality control, respectively.

3.2 | RNA viruses

Neither EIV nor ERVs were detected from any of the enrolled horses. However, a higher percentage of the nasal swab samples submitted failed quality control for the RNA viruses (25.1%, 42/167) than the DNA viruses (6.6%) and *S. equi* (7.2%). None of the horses that failed quality control had clinical signs consistent with equine influenza or equine rhinitis virus infection. There was a seasonal bias to the quality control failure with the late summer, early autumn months having the highest percentage failure (July 44.4%, August 42.9%, September 73.7%, and October 72.6%). Quality control failure rate was <20% for all the other months. The summer and fall months in Davis, CA are

TABLE 2 Hematologic parameters—groups are defined as PCR-positive nasal swab/respiratory disease clinical signs positive (PCR+/CS+), PCR-negative nasal swab/respiratory disease clinical signs positive (PCR−/CS+), PCR-positive nasal swab/respiratory disease clinical signs negative (PCR+/CS−), and PCR-negative nasal swab/respiratory disease clinical signs negative (PCR−/CS−)

Parameter Mean (SD)	PCR+/CS+ (n = 31)	PCR−/CS+ (n = 56)	PCR+/CS− (n = 19)	PCR−/CS− (n = 26)	One-way ANOVA
Hematocrit (%)	35.2 (4.2)	35.8 (4.3)	37.8 (2.7)	35.2 (4.0)	$P = .11$
White blood cells/μL	8598 (2100)	7901 (1984)	8021 (1557)	8275 (1489)	$P = .39$
Neutrophils/μL	5204 (1334)	5107 (1317)	5271 (1325)	5246 (1208)	$P = .95$
Lymphocytes/μL	2929 (1466)	2364 (961)	2378 (719)	2605 (766)	$P = .092$
Monocytes/μL	340 (210)	295 (109)	252 (112)	296 (107)	$P = .18$
Fibrinogen (mg/dL)	281 (120)	267 (102)	258 (84)	269 (84)	$P = .80$

typically hot and dry with presence of high levels of dust and particulate matter in the air.

3.3 | *Streptococcus equi* subspecies Equi

Streptococcus equi was not recovered by qPCR from any nasal secretions. However, 12/167 horses failed quality control, including one horse with enlarged submandibular lymph nodes. One horse with fever and enlarged submandibular lymph nodes had multiple nasal swabs taken during the quarantine period and all came were negative for *S. equi*; however, it was reported to be bacterial culture positive from a lymph node aspirate performed after release from quarantine.

3.4 | Hematologic variables

There were no significant differences in mean hematocrit ($P = .11$), WBC ($P = .39$), neutrophil count ($P = .95$), lymphocyte count ($P = .092$), monocyte count ($P = .18$), or fibrinogen ($P = .80$) between the four groups of horses as previously defined (Table 2).

4 | DISCUSSION

Our study demonstrated that equine herpesviruses are frequently detected in horses recently imported into the United States after long distance travel. Recognized equine respiratory herpesviruses were detected by qPCR in 52.1% (87/167) of the study horses this included EHV-2 (28.7%), EHV-5 (40.7%), and EHV-1 or EHV-4 in 4.2%. The EHV-1 qPCR-positive horses all displayed clinical signs of respiratory disease. Horses that were qPCR-positive for EHV-2, EHV-4, and EHV-5 did not predictably exhibit clinical signs of respiratory disease. None of the study horses were found to be qPCR-positive for EIV, ERAV, ERBV, or *S. equi*.

Our study found that γ-herpesviruses were commonly shed by large numbers of horses and that α-herpesviruses were shed by only 4.2% of the horses. The role γ-herpesviruses play in the development of respiratory disease has remained poorly understood. Equine herpesvirus-2 has been associated with outbreaks of upper respiratory tract disease in young horses[9], but EHV-2 has also been isolated from clinically normal horses.[10] Equine herpesvirus-5 has been associated with the development of multinodular pulmonary fibrosis (EMPF),[11] herpetic ocular disease,[12] and lymphoma[13] in horses, however, these diseases are rare while of EHV-5 shedding in the equine population is common.[14] It has previously been reported that horses with nasal secretion viral loads of >650 000 gB genes/million cells were more likely to have EMPF than controls.[15] Interestingly, 38.2% of EHV-5 qPCR positive horses in our study were shedding >650 000 gB genes/million cells. This elucidates the importance of clinical findings on interpretation of diagnostics and demonstrates that other factors such as travel stress and host-related factors are likely to play a role in the magnitude of viral shedding.

Our study showed that Friesian horses were more likely to shed EHV-4 than European warmbloods. A previous study looking at increased rectal temperature in horses during the first 48 hours of quarantine at US air import facilities found that Friesians were significantly more likely than warmbloods to have an increased rectal temperature during that time period.[16] Friesians have been shown to have a higher inbreeding coefficient that most other equine breeds including European warmbloods.[17,18] Inbreeding has been associated with increased susceptibility to disease in many species. In humans, increased susceptibility to herpes simplex virus-1 infection has been demonstrated to be heritable.[19]

Our study only had three foals; however, all were qPCR-positive for herpesvirus and were shedding very high viral numbers. Further investigation of viral shedding in transported foals is warranted. Previous surveillance studies in nontransported foals reported that <25% of the nasal swabs were found to contain herpesviruses.[20]

Equine influenza virus and ERVs were not detected in any horses in our study; however, 27.5% (46/167) of the samples failed quality control. Reasons for quality control failure may have been associated with inhibition within the sample, inadequate amount, or quality of genomic material, or DNA/RNA degradation. Inhibition within the sample can be caused by organic material that may obscure qPCR analysis, compounds that can inhibit the reactions (eg, cell lies) or bind genomic material (RNA/DNA).[21] The seasonal distribution of quality control failure is suggestive that there are environmental factors that may contribute to the inhibition. The months with the highest failure of quality control are the driest, dustiest, and warmest months in the study location, likely leading to considerable contamination of the nasal passages with organic debris and dust. It would be expected that inadequate

genomic material as a cause of quality control failure should not have a seasonal distribution. All of our samples were kept refrigerated in a viral culture media before processing to try to reduce DNA/RNA degradation; however, RNA is more sensitive to degradation than DNA. While we had a fairly high percentage of samples fail quality control, none of those horses had clinical signs typically associated with EIV, ERAV, or ERBV infection such as cough, fever, or tachypnea.

No horses were positive for *S. equi*, although 12 failed quality control. Of the horses that failed quality control, one had clinical signs consistent with strangles (enlarged lymph nodes and nasal discharge). In a clinical setting, it would have been prudent to collect a second sample for testing. Additionally, there was a single horse with consistent clinical signs, found to be negative on multiple nasal swabs, which was later found to be culture positive from a lymph node aspirate. It is currently recognized that nasal swabs are not as sensitive for recovery of *S. equi* as guttural pouch lavage or pharyngeal wash samples.[22,23] It is recommended that in clinical cases with consistent signs the type of sample collected should be adjusted to maximize the chances of recovery.

In our study, complete blood count was not useful in the identification of horses shedding herpesviruses with or without the presence of clinical signs. It has previously been reported that horses with EHV-1 infections had an elevation in absolute monocyte count within the first 5 days of disease and increased neutrophil to lymphocytes ratios early in disease followed by a decrease later in the course of disease.[24] It may be that hematologic measurements over time may be more helpful in linking respiratory signs to a viral infection.

The study had several limitations. First the study population was only composed of mares and stallions 2 years of age and older, and a few nursing foals entering the CEM quarantine facility. Geldings imported from outside the United States and weaned fillies and colts <2 years or age were not available for testing as they directly enter the United States from the port of entry quarantine facility. The proposed study horses represent $\sim$20% of all horses imported through the USDA import facility located in Los Angeles. Based on the results of horses <2 years of age in our study this may represent a higher risk population that the general population of imported horses. Horses <2 years of age have been shown to be significantly more likely to have an increased rectal temperature than horses >2 years of age after arrival at an air import facilities in the United States.[16] Further investigation of respiratory pathogen shedding by young imported horses is warranted.

Additionally, because sampling took place at a secondary quarantine site the horses were sampled $\geq$72 hours after arrival in the country. It is possible that we missed horses that were shedding during the initial three days after air transport. We do not know if there is a difference in air versus overland travel for transmission of respiratory pathogens in horses. There is some evidence to suggest that horses are more stressed in air travel based on heart rate and heart rate variability.[25] In humans, air transportation appears important in influenza propagation and it is likely that ground transport systems and transport hubs play a role in influenza propagation.[26]

Another limitation relates to the number of study horses. There is minimal data showing the expected frequency of shedding of common and lesser-characterized respiratory pathogens from horses recently imported into the United States. Equine herpesvirus type-1 shedding via nasopharyngeal swabs or blood was found in only 1% of horses in a population very similar to our study. Studies investigating the shedding of common respiratory pathogens in healthy horses attending various equine events or sales have shown 2–4% of horses shedding at least one of the respiratory pathogens.[1,2,26]

Imported horses traveling long distances represent a considerable risk for spreading infectious respiratory pathogens into horse populations. Herpesviruses are commonly shed by imported horses, and young horses and some breeds may represent a higher risk. It is important to note that clinical signs were poorly correlated with being qPCR-positive for EHV-2, EHV-4, or EHV-5. The lack of clinical signs in horses with herpes viral infection increases the risk of introducing a shedding horse into a susceptible population. These findings demonstrate the need for rigorous biosecurity measures when introducing recently imported horses into resident equine populations.

ACKNOWLEDGMENTS

The authors thank the University of California Veterinary Medical Teaching Hospital Equine Field Service for collection and submission of samples and to the laboratory staff of the University of California, Davis Real-time PCR Research, and Diagnostics Core Facility for performing analyses of submitted samples. This project was supported by a resident grant from the University of California, Davis Center for Equine Health with funds provided by the State of California satellite wagering fund and contributions by private donors. Portions of this study were presented at the 2017 American College of Veterinary Internal Medicine Forum, National Harbor, MD.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Our study was approved by the IACUC Committee of the University of California, Davis.

ORCID

Fauna Leah Smith http://orcid.org/0000-0001-5535-7726

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How to cite this article: Smith FL, Watson JL, Spier SJ, et al. Frequency of shedding of respiratory pathogens in horses recently imported to the United States. *J Vet Intern Med*. 2018;32:1436–1441. https://doi.org/10.1111/jvim.15145